Archaebacterial reverse gyrase cleavage-site specificity is similar to that of eubacterial DNA topoisomerases I

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ABSTRACT

ATP-dependent type I topoisomerases from extremely thermophilic archaebacteria – reverse gyrases – drive positive supercoiling of DNA. We showed that reverse gyrase from *Desulfurococcus amylolyticus* breaks the DNA at specific sites and covalently binds to the 5′ end. In 30 out of 31 sites located in pBR322 DNA fragments, cleavage occurs at the sequence 5′—CNNN|— (N is any base). The same rule was previously shown to hold for single-stranded DNA breakage by eubacterial topoisomerases I. The relative cleavage frequencies at different sites depend on Mg^{2+} and temperature. We discuss the possible physiological and mechanistic role of the above specificity of the bacterial topoisomerases I.

INTRODUCTION

DNA topoisomerases play a dual role in the cell (1–9). On the one hand, they catalyze the passage of one DNA segment through a transient break in another segment and change DNA linking number thereby. On the other hand, they take part in the structural organization of the cell genome and of the processes it is involved in.

In the topological domains of DNA the translocation of RNA polymerases, a replisome or junctions along the chain is coupled with the rotation of separate segments of the double helix, which is tantamount to the movement of linkings from one end of a segment to the other (4–13). The topoisomerases situated at the boundaries of these segments bring the linkings in at one end and out at the other. The size of the rotating stretches, the distribution of stress along them, and the rate of the entire process are essentially dependent on the specific sites the topoisomerases work at and on their topoisomerization rates. Thus, DNA topoisomerases are the elements of an integral dynamic system.

There is little knowledge about how topoisomerases choose the sites in the DNA to carry out their functions in the cell. It is clear though that DNA conformation and sequence play a significant role in the DNA-protein interactions. In addition to the classification according to type (I or II), topoisomerases can be divided with respect to the conformation of substrate DNA: the first group includes enzymes operating on duplex DNAs, and the second consists of enzymes which use single-stranded regions. The reverse gyrase of extremely thermophilic archaebacteria, along with other bacterial type I topoisomerases, belongs to the second group (3, 4, 14–18).

In contrast with the first group, the cleavage-site specificity of the second group topoisomerases has proved to be rather simple: the presence of a cytosine four nucleotides away on the 5′ side of the break (position −4) is observed in the overwhelming majority of the sites mapped for DNA topoisomerases I from *Escherichia coli* (*Eco*) and *Micrococcus luteus* (*Mlu*) (19–21). The present paper reports a study on the cleavage-site specificity of reverse gyrase from the extremely thermophilic sulfur-reducing anaerobic archaebacterium *Desulfurococcus amylolyticus* (22–24).

MATERIALS AND METHODS

Enzymes, DNA and chemicals

The isolation of reverse gyrase from *Desulfurococcus amylolyticus* (*Dam* reverse gyrase) was described in (24). Restriction endonucleases *Eco*RI, *Msp*I, T4 polynucleotide kinase were purchased from NPO Ferment (Vilnius, USSR), bacterial alkaline phosphatase, Klenow fragment of *E. coli* DNA polymerase I were from Pharmacia, proteinase K was from Sigma. The pBR322 DNA containing over 90% in the superhelical form was obtained from NPO Ferment. [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from NPO Isotop (Tashkent, USSR). Sephadex G-50 was obtained from Pharmacia, spermidine-3HCl was from Serva, glycerol was from Fluka, polyacrylamide was from BDH, other chemicals were from Sigma.

Preparation of end-labelled DNA fragments

The pBR322 DNA was linearized with *Eco*RI, dephosporylated with bacterial alkaline phosphatase and labelled with the aid of

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T4 polynucleotide kinase using [γ-^{32}P]ATP (25). The DNA was then cut with *Msp*I. Two resulting 5′-end-labelled fragments (1−161 and 4361−3900, numbered according to (26)) were isolated by preparative gel electrophoresis (25). The 3′-end-labelled DNA was prepared from the 5′-end-labelled fragment that was dephosphorylated and its 3′ end (resulting from *Msp*I cutting) was filled by Klenow fragment and [α-^{32}P]dCTP. The DNA was transferred into required buffer by gel filtration on Sephadex G-50 microcolumns (25).

DNA cleavage assays

DNA and *Dam* reverse gyrase were incubated in 10 to 20 μl of 50 mM Tris-HCl (pH 7.5 at 25°C), 100 mM NaCl, 5 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 1 mM spermidine-3HCl, 1 mM dithiothreitol at 90°C for 1 min. When indicated, 1 mM ATP was added. The reaction was stopped by addition of hot SDS to 1%. The termination of the reaction by hot NaOH was done as in (19). Where indicated, the reaction was stopped by addition of hot EDTA to 10 mM. Incubation of 3′-end-labelled fragment with proteinase K (400 μg/ml) was at 40°C for 40 min, followed by extraction with phenol/chloroform (50:50). Base-specific chemical cleavage reactions were as in (27).

Gel electrophoresis and data processing

Gel electrophoresis in 1% agarose was done as described (24).

Ethanol precipitates of labelled DNA were washed twice with 70% ethanol. Following light vacuum drying, pellets were dissolved in 3 μl of deionized formamide with bromophenol and xylene cyanol, heated and electrophoresed in a 6% polyacrylamide gel with 8 M urea (27). The aliquots to be applied on the gel had nearly equal radioactivity. After electrophoresis, gels were autoradiographed on RT-1 film (Tasma, USSR) without intensifying screens.

The autoradiographs were scanned by a laser Ultroscan XL densitometer (LKB). The densitometry results were processed at IBM PC/XT with the aid of the LKB GSXL software.

RESULTS

Covalent binding of *Dam* reverse gyrase to the 5′ end of DNA

In the course of topological reactions, DNA topoisomerases, both from eubacteria and eukaryotes, transiently break the DNA strand(s) and bind covalently to one of its (their) ends. The exposure to protein denaturants, such as alkaline or SDS, irreversibly fixes the intermediate covalent complex of topoisomerase and DNA. The analysis of such complexes using DNA labelled at one of the ends has shown that type I DNA topoisomerases from eubacteria bind covalently to the newly formed 5′-phosphoryl termini of DNA whereas that from eukaryotes binds to the 3′-phosphoryl termini (16, 28−30).

Proceeding from this scheme, we have analyzed which of the DNA ends is the one that archaebacterial type I topoisomerase − *Dam* reverse gyrase − is attached to. Figure 1 (lanes 1−3) shows the results of gel electrophoresis of superhelical pBR322 DNA after incubation with *Dam* reverse gyrase without ATP and upon addition of SDS to the mixture at 90°C. One can see that the fraction of DNA in form II increases and in form I decreases in the enzyme-quantity-dependent manner. The same result was obtained when NaOH was used instead of SDS (Fig. 1, lanes 4−6). DNA cleavage was not observed if the reaction was terminated by EDTA (Fig. 1, lane 7). Reverse gyrase retains its supercoiling activity after incubation without ATP at 90°C for 1 min as was demonstrated by subsequent addition of ATP (Fig. 1, lane 8). Thus, single-strand breaks are induced in the DNA molecules through the denaturation of reverse gyrase by SDS or alkaline.

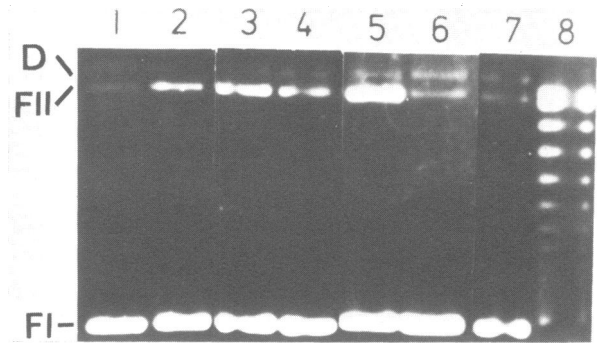


Figure 1. Agarose gel electrophoresis of superhelical pBR322 DNA (0.1 μg) after incubation in the absence of ATP with 12 ng (lanes 2, 4, 8), 50 ng (lanes 3, 7), or 70 ng (lane 5) of *Dam* reverse gyrase, or without it (lanes 1, 6) at 90°C for 1 min and subsequent addition of hot SDS to 1% (lanes 1−3), or NaOH as in (19) (lanes 4−6), or EDTA to 10 mM (lane 7). In lane 8, ATP was added to 1 mM and incubation was continued at 90°C for next 10 min and stopped by EDTA. The positions of the form I (FI), form II (FII) DNA and of supercoiled dimers (D) are indicated.

In the next experiment, *Dam* reverse gyrase was incubated with the *Eco*RI/*Msp*I fragment (460 bp) labelled with ^{32}P either at the 5′ or at the 3′ end (bottom strand). After the addition of SDS and electrophoresis under denaturing conditions a number of the shorter 5′-end-labelled fragments appeared in the gel (Fig. 2, lane 4). In contrast, after the addition of SDS practically all 3′-end-labelled DNA did not enter the gel; however, the treatment of this sample with proteinase K released a number of shortened 3′-end-labelled DNA fragments (Fig. 2, lanes 1, 2).

Thus, the SDS-treatment of the complex of reverse gyrase and DNA generates a single-chain scission in the DNA, and the enzyme is found linked covalently to the 5′ end of the scission (31, 32).

Sequence specificity of *Dam* reverse gyrase cleavage

To test the specificity of cleavage by *Dam* reverse gyrase on duplex DNA substrate, 161- and 460-bp *Eco*RI/*Msp*I fragments of the pBR322 labelled with ^{32}P at *Eco*RI-generated 5′ ends (top and bottom strands, respectively) were used. Each fragment was incubated with reverse gyrase at 90°C for 1 min and then hot SDS was added. The nucleotide sequence for a portion of the 161-bp fragment used in the above experiment and the locations of break sites are shown in Fig. 3. The sites are marked with vertical lines above the nucleotide 5′ of cleavage.

We have located 16 cleavage sites within the 161-bp fragment and 15 sites within the 460-bp fragment (Table 1). Following (21) we tried to derive the consensus sequence around *Dam* reverse gyrase cleavage sites. Figure 4A shows the number of times a nucleotide appears at a particular position relative to the location of the cut site. The most striking result is that the nucleotide four bases 5′ to the cut (position −4) is a cytosine residue 30 out of 31 times.

It is surprising that the same rule was previously shown to hold for single-stranded DNA cleavage by *Eco* and *Mlu* topoisomerases I in 90−97% of events (19−21). However, we should note that another type I topoisomerase, *Eco* topoisomerase

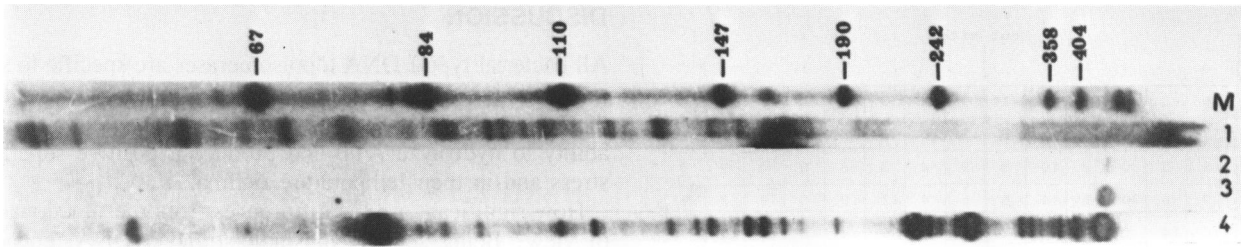


Figure 2. Polyacrylamide gel electrophoresis in denaturing conditions of cleavage products of 3'-end-labelled 460-bp *Eco*RI/*Msp*I fragment of pBR322 DNA (lane 3) upon incubation with *Dam* reverse gyrase (7.5 ng DNA and 50 ng enzyme) and subsequent treatment with hot SDS (lane 2) followed by the treatment with proteinase K (lane 1). Lane 4, the same as in lane 2, but 5'-end-labelled fragment (6 ng DNA) was used. Lane M, 3'-end-labelled *Msp*I digest products of pUC19 DNA.

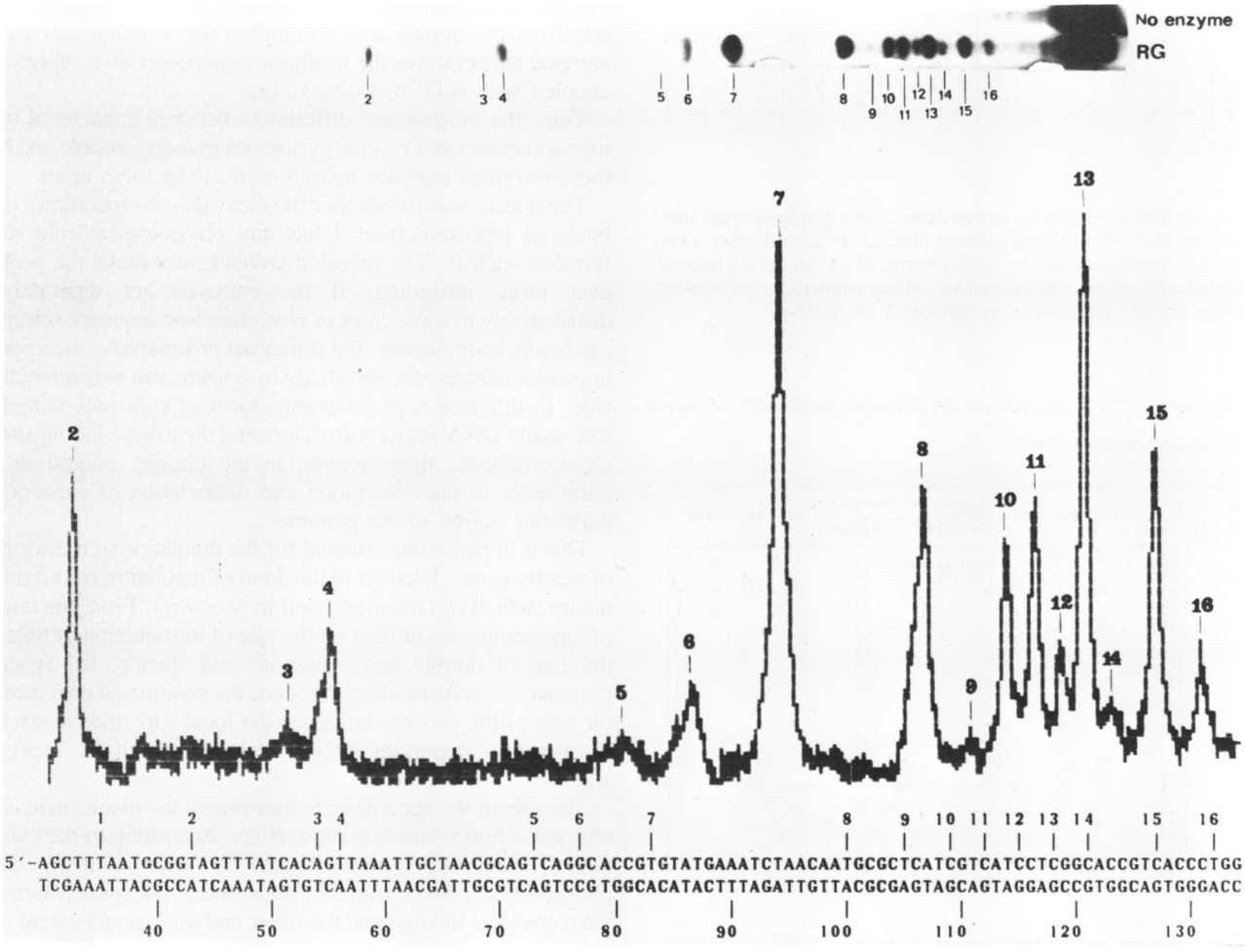


Figure 3. The densitometric pattern of cleavage of 160-bp *Eco*RI/*Msp*I fragment of pBR322 DNA by *Dam* reverse gyrase. The nucleotide sequence read off the autoradiograph is shown below; the sites of cleavage yielding the enumerated bands shown in the tracing are indicated above the sequence enumerated accordingly.

III, does not apparently share this rule (33). Unfortunately, we have no statistics on topoisomerase III-mediated DNA breaks at our disposal.

In addition, Dean and Cozzarelli (21) searched the *Eco* topoisomerase I sites for a sequence peculiarity near C residues that are not associated with a cut. The 18% of sites with the relevant C residue positioned at −4 were not associated with cleavage and contained often a G residue at position −1 or a C residue at position −5. We carried out a similar analysis for *Dam* reverse gyrase (Fig. 4B) but found no such correlation.

Since we used practically the same pBR322 DNA fragments as those used by Tse *et al.* (19), we took a chance to compare the positions and relative susceptibilities of cleavage sites for *Eco*, *Mlu* and *Dam* topoisomerases I (Table 1). As it follows from the Table, one can hardly choose a pair among the three enzymes that would have many more coinciding sites than the other pairs. This is surprising in view of the phylogenetic distance between *D. amylolyticus* on the one hand and *E. coli* and *M. luteus* on the other (34, 35).

Using the 161-bp fragment, we investigated the way the DNA cleavage by reverse gyrase depends on ATP, Mg^{2+} and temperature. Figure 5 clearly shows that cut positions do not

A

cut site

	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8
A	8	12	7	6	1	13	5	8	11	13	8	10	6	8	9	8
G	7	3	5	5	0	8	10	5	9	4	3	8	5	4	9	5
C	4	8	9	6	**30**	6	4	6	6	4	11	9	5	9	3	9
T	12	8	10	14	0	4	12	12	5	10	9	4	15	10	10	9

Total sequences = 31

B

	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8
A	7	7	8	6	0	5	4	4	4	9	6	6	7	4	5	7
G	7	5	6	6	0	4	3	5	7	3	5	4	8	7	7	4
C	5	6	5	7	**23**	6	10	7	4	3	4	5	5	3	6	3
T	4	5	4	4	0	8	6	7	8	8	8	8	3	10	10	9

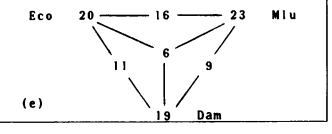
Total sequences = 23

Figure 4. **A**, nucleotide frequencies around *Dam* reverse gyrase cleavage sites in 161- and 460-bp *Eco*RI/*Msp*I fragments of pBR322. **B**, sites at which a cut was not observed but was expected due to the presence of a C residue at a position −4 are aligned with reference to that residue. Minus positions are nucleotides 5′ of cleavage and plus positions are nucleotides 3′ of cleavage.

Table 1. Relative cleavage frequencies at different sites by *E. coli*[a], *M. luteus* and *D. amylolyticus* topoisomerases I

Region of pBR322 examined[b]	Position of cleavage[c]	Eco	Mlu	Dam
Bottom strand, 4170–4330	4174	2	30	4
	4177		3	
	4188			6
	4195	19		4
	4201			4
	4206			10
	4207	33	5	
	4218		3	10
	4222			8
	4229			6
	4245		25	
	4246	3	45	15
	4247		37	
	4248		17	
	4253	55	41	9
	4255		26	
	4267		20	3
	4272	65	18	
	4274	67	35	
	4281	47	15	100
	4286	47	24	
	4290	29	23	8
	4291	44	15	
	4307			19
	4327	7	19	
	4328	100	100	43
	4329	13	28	
	4330	11	3	

Region of pBR322 examined[b]	Position of cleavage[c]	Eco	Mlu	Dam
Top strand, 31–140	35		15	72[d] 35
	38		29 38	
	43		100	100 100 49
	54		18	12 6
	56		25	40 20
	73			4
	77			14
	83			92
	100			45
	105			2
	109			33
	112			49
	115			20
	118			100
	121			8
	127			57
	132			14



[a]The cleavage positions and relative cleavage frequencies at different sites by *E. coli* and *M. luteus* have been taken from the paper of Tse *et al.* (19).
[b]The sequence is numbered according to Watson (26).
[c]The position of cleavage refers to the nucleotide on the 5′ side of the cut.
[d]The efficiencies of reverse gyrase breaks in the region from 31 to 60 was normalized to the maximum one in this region. The region from 60 to 140 has not been examined in the paper of Tse *et al.* (19).
[e]'Preference triangle' shows the number of breaks in compared regions for each topoisomerase (vertices), the number of coinciding sites for each pair (sides), and for all three enzymes

change. At the same time, the intensities of some bands depend on temperature and the presence of Mg^{2+} but not of ATP. We should note for comparison that ATP greatly affects the double strand DNA cleavage reaction and does not affect the single strand cleavage reaction by ATP-dependent type II DNA topoisomerases (36–40).

DISCUSSION

All bacterial type I DNA topoisomerases are specific to single-stranded DNA, and *Eco*, *Mlu*, and *Dam* topoisomerases I share the −4C rule of cleavage. The enzymes differ, however, in their ability to hydrolyze ATP and produce a positive superhelical stress and in their temperature optima.

How significant are the differences from the functional point of view? Being specific to single-stranded DNA, eubacterial relaxing type I topoisomerases, like reverse gyrases, introduce linkings into DNA but fail to eliminate positive supercoils. The same specificity ensures a feedback between the topoisomerization activity and the stability of the DNA duplex: if the introduction of linkings is not compensated by other processes, the resulting increase in the superhelical stress (decrease in the negative stress) stabilizes the duplex and diminishes the winding activity. At extreme temperatures the feedback requires positive supercoiling coupled with ATP hydrolysis (18).

Thus, the biochemical differences between eubacterial type I topoisomerases and reverse gyrases seem indispensable and bring these enzymes together instead of drawing them apart.

The question as to whether the cleavage-site specificity of the bacterial topoisomerases I has any physiological role so far remains unclear. The revealed coincidences make the problem ever more intriguing. If the enzymes act separately or distributively in some cases *in vivo*, their low sequence selectivity can hardly be important. But if they act processively, incorporated in protein clusters, the specificity to cytosine can play a functional role. In this case a given arrangement of cytosines within the low-stable DNA region can determine the exact binding sites of topoisomerases, their amount in the cluster, conditions and parameters of the association and dissociation of clusters in a particular region of the genome.

This is in particular essential for the regulation of transcription of nearby genes. Peculiar to this kind of regulation is its dynamic nature, which can manifest itself in two ways. First, the amount of topoisomerases reflects on the rate of introduction of linkings, the rate of double helix rotation, and, hence, the speed of polymerases translocation. Second, the position of enzymes and the rate of the process influence the local superhelical stress in functionally important DNA regions, which is especially significant for promoters.

The role of the specificity to cytosine in the mechanism of the enzyme action seems less mysterious. According to the existing model, bacterial topoisomerases I, after cleaving DNA phosphodiester bond, trap both ends, the 5′-phosphoryl terminus via a covalent linkage and the other end via a noncovalent bond (21, 29, 41–44). The base of cytosine at position −4 appears to form the tight contact with the enzyme. The anchorage of the base is essential for preventing this DNA end from slipping. The maintenance of such a contact in the interval between successive cycles may be important for the processivity.

The mechanism of the gate formation to let the second DNA segment through is not specified yet. Two extreme cases are plausible: moving apart of the DNA-protein trapping contacts if the protein skeleton is flexible enough, or the strict fixation of the distance between the contacts by protein frame and turning aside of the segment confined between the noncovalent contact with the base and the loose 3′-terminus of the cleaved strand. In the latter case, the minimum size of the turning segment is limited by the transverse dimensions of the segment to be pulled through. The size of 4 nucleotides is enough for a single strand

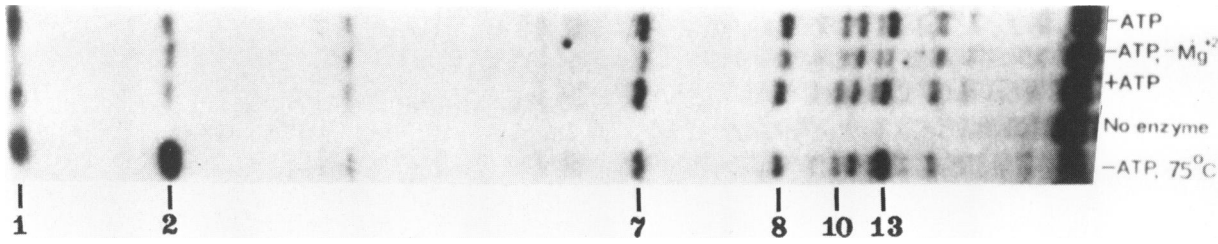


Figure 5. Polyacrylamide gel electrophoresis in denaturing conditions of cleavage products of 160-bp *Eco*RI/*Msp*I fragment of pBR322 DNA (2.5 ng) upon incubation with *Dam* reverse gyrase (25 ng) at different assay conditions and subsequent treatment with hot SDS. Lane without ATP corresonds to standard conditions; bands with considerable changes in intensity are enumerated according to Fig. 3.

and possibly a double helix to pass if it is turned appropriately (with its minimum transverse dimension along the line between the contacts). The motility of the contacts is important at the rejoining step. This quantity, and the length of the swinging segment, may tell on the characteristic time required for the 3′-terminus to come across the active centre.

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